

THE ROLE OF TYPE V SECRETION AUTOTRANSPORTERS IN THE VIRULENCE OF *XYLELLA FASTIDIOSA*

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ABSTRACT

Autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (passenger domain) across the outer membrane of Gram-negative bacteria. Members of this protein family have been identified as rational targets for the design of novel vaccines directed against Gram-negative pathogens (Wells et al., 2007). Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xylella fastidiosa* Temecula 1 (*Xf*-PD). During the period under review, we have completed our construction of strains containing single or multiple mutations in these genes and are examining how the absence of one or more autotransporters affect *Xf* cell physiology and virulence. We have shown that two of the autotransporters (PD0528 and PD1379) are present on the bacterial cell surface and are involved in autoaggregation and biofilm formation. Studies in grapevines indicate that the absence of either PD0528 or PD1379 reduces the virulence of *Xf* in grapevines. We have also initiated experiments to characterize PD0218, PD0313, and PD0950, the three autotransporters predicted to have proteolytic activity. Strains carrying a mutation in either PD0218 or PD0950 exhibit a hypervirulent phenotype in grapevines. In contrast, grapevines infected with a strain missing all three proteases exhibit symptoms much later than wildtype. Comparisons of the secreted proteins from the three single mutants suggest that each protease has a different set of target proteins. Experiments are currently underway to identify the protein targets of the individual proteases and the virulence factors that require these proteases for their maturation. The ultimate goal is to develop methods for interfering with this maturation, thereby reducing the virulence of this important plant pathogen.

LAYPERSON SUMMARY

Autotransporters are virulence proteins that are found on the surface of many bacterial pathogens. These proteins have been identified as rational targets for the design of novel vaccines and control strategies. The goal of this project is to characterize the six autotransporters of *Xylella fastidiosa* (*Xf*). Four of these proteins have enzymatic activity. It seems likely that strategies developed to disrupt the function of these proteins in other systems will also be effective against the *Xf* proteins. The remaining two proteins are unique to *Xf* and appear to be involved in the attachment of the bacterium to solid surfaces. Treatments designed to prevent this attachment could have a profound impact on the ability of *Xf* to cause Pierce's Disease.

INTRODUCTION

The causative agent of Pierce's disease (PD) is the Gram-negative bacterium, *Xylella fastidiosa* (*Xf*) [for a recent review, see (Chatterjee et al., 2008)]. An important feature of the *Xf* infection cycle is the ability of this pathogen to colonize and interact with the xylem tissue of susceptible plants and with the foregut of insect vectors. Successful establishment of the pathogen is dependent on the ability of *Xf* to acquire essential nutrients, to adhere to the host cell surfaces, and to escape any host defense mechanisms. Comparison of the *Xf*-PD genome to other bacterial pathogens has resulted in the identification and characterization of a number of genes that are potential virulence factors. Many of these virulence determinants are proteins that are either secreted to the bacterial cell surface or released into the external environment (Dautin and Bernstein, 2007; Henderson et al., 2004). Our work has focused one category of virulence determinants, the AT-1 autotransporters.

AT-1 autotransporters are dedicated to the secretion of a single specific polypeptide, the passenger domain, across the outer membrane. Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xf*-PD. Functional sequence predictions indicate that three of these secreted proteins have proteolytic activity (PD218, PD0313, PD0950), one protein has lipase/esterase activity (PD1879), and two of the proteins encode tandem repeats of a 50-60 amino acid motif that is only found in *Xf* species (PD0528, PD1379). The goal of this project is to establish the role of these secreted proteins in *Xf* cell physiology and pathogenicity. Given the importance of AT-1 proteins in the virulence of other Gram-negative pathogens, it is highly likely that most of the *Xf*-PD AT-1 proteins will play a role in *Xf* virulence. Thus, characterizing of these proteins and identifying methods for disrupting their function should allow the development of strategies that impact the ability of *Xf* to colonize plant tissue and to initiate the PD disease cycle in susceptible grapevines.

OBJECTIVES

1. Generate a mutation in each of the six AT-1 genes and determine their impact on *Xf* cell physiology and virulence. The construction of strains carrying double and triple mutations in the various autotransporters is also part of this objective.
2. Examine the biochemical properties and location of the six AT-1 passenger domains.

RESULTS AND DISCUSSION

Characterization of the AT-1 autotransporters with *Xf*-species specific passenger domains:

Two of the most interesting *Xf*-PD autotransporters are PD0528 and PD1379. The passenger domains of these proteins encode tandem repeats of a 50-60 amino acid motif that is only found in *Xf* species. PD0528 has six repeats, whereas PD1379 has three repeats. In addition, both passenger domains contain WD40 repeats. WD40 repeats are predicted to create a specific structure, a β propeller-like platform (Hudson and Cooley, 2008). In other systems, binding partners associate with this platform either stably or reversibly. Usually, the binding partner is a protein that recognizes a specific consensus binding motif within the β -propeller. Therefore, the presence of WD40 repeats makes it highly likely that the passenger domains of PD0528 and PD1379 are each interacting with a specific protein or set of proteins.

We have created a number of strains and tools that have facilitated our analysis these proteins. Specifically, we have generated strains containing null mutations in either PD0528 or PD1379 and a double mutant that eliminates both proteins. The resulting mutants exhibit decreased autoaggregation and biofilm formation *in vitro*. Interestingly, grapevines infected with either the double mutant or the PD0528::Cm mutant do not exhibit PD symptoms. The major difference is that the double mutant can only be recovered close to the site of inoculation, whereas the PD0528::Cm mutant can be recovered 12 cm above the inoculation site. In contrast, grapevines infected with the PD1379::Gm mutants still exhibit some PD symptoms. However, the symptoms are not as severe as those observed with the wildtype Temecula1 control. To confirm these phenotypes, we are in the process of constructing a series of strains for complementation analysis. We have also generated antibodies against the passenger domains of PD0528 and PD1379 and have used these antibodies in localization studies. Based on immunofluorescence microscopy, the passenger domains of both proteins are exposed on the *Xf* cell surface. Furthermore, fractionation studies indicate that PD0528 is an extremely abundant protein in the *Xf* outer membrane and can also be found in membrane vesicles and in the supernatant. In contrast, PD1379 is present at much lower levels and is only found in the outer membrane. The difference in the localization patterns of PD0528 and PD1379 suggest that these autotransporters may be involved in different aspects of the PD infection cycle.

We have also expressed both PD0528 and PD1379 on the surface of the *E. coli* strain UT5600. UT5600, which is deficient in the outer membrane proteases OmpT and OmpP, is commonly used for autodisplay (also known as live-cell surface display) (Jose and Meyer, 2007). Although the expression levels were low, both proteins were localized to the *E. coli* outer membrane and conferred the ability to autoaggregate and to form a biofilm. The ability of PD0528 and PD1379 to confer new phenotypic properties to *E. coli* indicates that these proteins are directly responsible for the observed traits. Moreover, the fact that these proteins are functional and present in the *E. coli* outer membrane has allowed us to perform preliminary tests concerning how these autotransporters are secreted. Genetic analysis in *E. coli* has established that the secretion and correct folding of most outer membrane proteins occurs through the BAM (β -barrel assembly machine) complex, which is composed of five proteins BamA-BamE (Knowles et al., 2009). There are orthologs to four of these proteins in *Xf*: PD0326 (BamA), PD1620 (BamB), PD1756 (BamD), and PD1375 (BamE). Our studies using the *E. coli* system suggest that the translocation and correct insertion of autotransporters into the *Xf* outer membrane is mediated by the *Xf* BAM complex. As shown in **Figure 1**, a mutation that disrupts the BAM complex (*bamB*::Km^R) interferes with the autoaggregation phenotype conferred to *E. coli* by the PD0528 protein (--■--vs --□--). A simple explanation for this result is that the PD0528 requires BamB for its localization to the *E. coli* outer membrane. It also implies that the mechanism for outer membrane protein localization is conserved between *E. coli* and *Xf*.

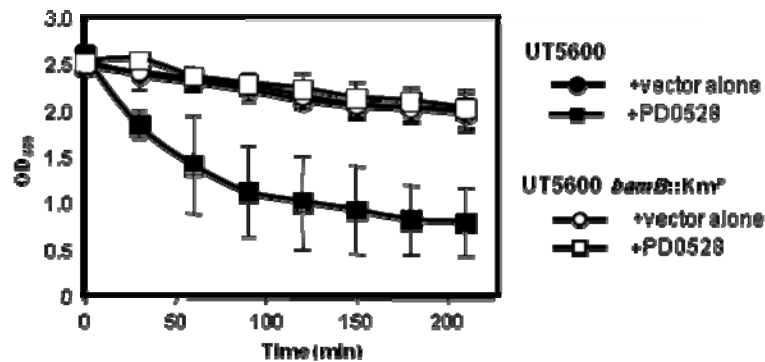


Figure 1. The autoaggregation phenotype conferred by PD0528 requires a functional BAM complex. At the beginning of the experiment, the *E. coli* cultures were adjusted to approximately the same optical density (OD₅₅₀) and vigorously shaken for 10 s. Samples were then taken ~0.5 cm from the top of the culture tube at the indicated times and the OD₅₅₀ was measured.

The serine protease AT-1 autotransporters:

PD0218, PD0313, and PD0950 are members of the phylogenetic clade containing the S8 subtilisin-like serine protease autotransporters (Tripathi and Sowdhamini, 2008). Members of this family have been implicated in defense, growth on proteinaceous compounds, and the proteolytic maturation of virulence factors. Although many serine proteases have broad specificities, some are very specialized. One of the best studied members of this clade is the SphB1 autotransporter protein of *Bordetella pertussis* (Coutte et al., 2001). SphB1 serves as a specialized maturation protease, responsible for the timely maturation and extracellular release of the filamentous haemagglutinin FHA. One of the goals of this project is to determine the specificity and targets of PD0218, PD0313 and PD0950.

As a first step in this analysis, we have generated strains containing mutations in one, two, or all three of the AT-1 serine proteases.

These strains and some of their properties are listed in the following table (**Table 1**):

Table 1. The properties of the AT-1 serine proteases mutants.

Strain	AT-1 Mutation(s)	Biofilm formation in vitro	Week PD symptoms first appear*
Temecula	Wildtype	0.688 ±0.12	11
TAM147	PD0218::Cm ^R	0.536 ±0.08	8
TAM152	PD0313::Gm ^R	0.248 ±0.02	10
TAM146	PD0950::Em ^R	0.469 ±0.02	8
TAM148	PD0218::Cm ^R , PD0950::Em ^R	0.531 ±0.07	8
TAM150	PD0218::Cm ^R , PD0313::Gm ^R	0.479 ±0.09	8
TAM151	PD0313::Gm ^R , PD0950::Em ^R	0.580 ±0.07	10
TAM153	PD0218::Cm ^R , PD0313::Gm ^R , PD0950::Em ^R	0.633 ±0.11	14

* Three plants were inoculated for each mutant on 6/22/09. Disease severity was assessed weekly using the visual scale (0 to 5) described by Guilhabert and Kirkpatrick (2005). On this scale, healthy plants receive a score of 0. The table lists the week when the infected grapevines first receive a score of 1 (only one or two leaves with scorching symptoms starting on the margins of the leaves).

Our characterization of the single mutants suggests that the three serine proteases are involved in different aspects of *Xf* cell physiology and pathogenicity. The mutations in PD0218 and PD0950 result in reduced clumping in liquid and a slight decrease in biofilm formation. In contrast, the mutation in PD0313 eliminates clumping in liquid and has a more severe impact on biofilm formation. The PD0313 mutant also forms a confluent lawn on solid medium. When introduced into grapevines, all three mutants produced symptoms earlier than Temecula1. The phenotype observed for our PD0218 mutant is similar to the phenotype reported by Guilhabert and Kirkpatrick (2005) for a strain carrying a Tn5 insertion in this locus. In contrast, grapevines inoculated with the triple mutant TAM153 exhibited symptoms much later than Temecula1. We plan to continue monitoring disease progression every two weeks for a total of 32 weeks after inoculation. Then, experiments will be conducted to determine the bacterial population at various points above the inoculation site.

We have also conducted preliminary experiments testing the feasibility of two approaches to determine the targets of the proteases. One approach is to compare the protein composition of the outer membrane, the membrane vesicles, and the secretome of the single mutants to wildtype on SDS-PAGE gels stained with Syphro Ruby. An example of this type of analysis is shown in **Figure 2**. Based on MALD-TOF-MS analysis of the proteins in indicated band, the PD0218 secretome is missing bacteriocin, which is encoded by PD1427. Bacteriocins are known to contribute to the competitiveness of the producing organisms and have been identified as potential targets for alternative approaches for plant disease control (Holtmark et al., 2008). The PD1427 bacteriocin resembles the *Rhizobium leguminosarum* RTX (repeats in toxin) protein, a bacteriocin that is similar to hemolysin and leukotoxin (Machado et al., 2001). Subtilisin-like serine proteases are known to function as the maturation enzyme for the bacteriocin-like lantibiotics produced by some Gram-positive bacteria (Tripathi and Sowdhamini, 2008). Therefore, although more experiments are needed, the simplest explanation for our results is that PD0218 is required for the maturation of PD1427.

The first approach works well for abundant proteins, but is of limited usefulness for less highly expressed proteins. Our second approach will be to compare the protein composition of the outer membrane, the membrane vesicles, and the secretome of the single mutants to wildtype by Western analysis. The rate-limiting step for this approach will be the availability of suitable antibodies. In our initial experiments, we used the Anti-PD0528 and Anti-PD1379 antibodies. Although we did not detect any differences using Anti-PD0528 antibody, we obtained an extremely interesting result using the Anti-PD1379 antibody. As shown in **Figure 3**, we are unable to detect PD1379 in the outer membrane of the PD0313 mutant using Anti-PD1379 antibody. It is not clear why the absence of the PD0313 protease is impacting PD1379

localization to the outer membrane. Although we are considering a number of models, further experiments are needed to establish whether or not this is a direct or an indirect effect.

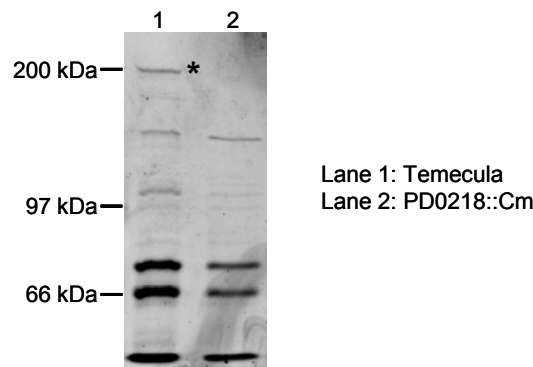


Figure 2. Proteins secreted by the PD0218 mutant. The secreted proteins from PD3-grown wildtype (lane 1) and PD0218 mutant (Lane 2) were concentrated using an Amicon centricon filter. The proteins were separated on a 6% SDS-PAGE gel and stained with Syphro Ruby. The band indicated by the star was excised and then analyzed by MALDI-TOF-MS at the UC Davis Molecular Structure Facility.

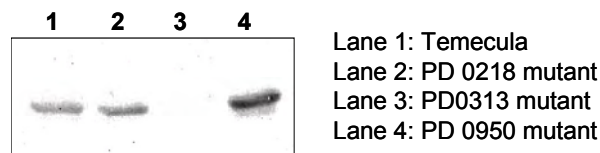


Figure 3: Western analysis of the outer membrane proteins using Anti-PD1379 antibody. The outer membrane proteins from wildtype and the protease mutants were separated on a 8% SDS-PAGE gel and then visualized by Western

Finally, we plan to examine how the protease mutants affect the maturation of hemagglutinin using antibodies prepared by Dr. Bruce Kirkpatrick (UC Davis). Based on analogy to SphB1 and its role in the maturation of filamentous hemagglutinin in *B. pertussis*, it is tempting to speculate that one of the three serine protease autotransporters will serve as a specialized maturation protease for *Xf*-PD hemagglutinin.

CONCLUSIONS

Autotransporters have been identified as rational targets for the design of novel vaccines and control strategies. The goal of this project is to characterize the six autotransporters of *Xf*. During the period under review, we have completed our construction of strains containing single or multiple mutations in these genes and are examining how the absence of one or more autotransporters affect *Xf* cell physiology and virulence. We have shown that two of the autotransporters (PD0528 and PD1379) are involved in autoaggregation and biofilm formation. Studies in grapevines indicate that the absence of either PD0528 or PD1379 reduces the virulence of *Xf* in grapevines. We have also initiated experiments to characterize the three autotransporters predicted to have proteolytic activity. Strains carrying a mutation in either PD0218 or PD0950 exhibit a hypervirulent phenotype in grapevines, whereas strain lacking all three proteases is less virulent than wildtype. Comparisons of the secreted proteins from the three single mutants suggest that each protease has a different set of target proteins. Experiments are currently underway to identify the protein targets of the individual proteases and the virulence factors that require these proteases for their maturation. The ultimate goal is to develop methods for interfering with this maturation, thereby reducing the virulence of this important plant pathogen.

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